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Dependence of upper limit of metastability on supersaturation in nephrolithiasis

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Dependence of upper limit of metastability on supersaturation in nephrolithiasis. Formation of renal stones requires supersaturation (SS) high enough to induce crystallization; such a SS is referred to as the upper limit of metastability (ULM). The ULM for calcium oxalate (CaOx) or calcium phosphate can be measured by adding oxalate or calcium to urine, respectively, and noting the point at which overt crystallization occurs as evidenced by clouding. In principle, the urine should be more prone to form stone crystals as its SS approaches the ULM, and the SS ULM distance has been used as an index of stone forming potential. In addition, one would expect the ULM and initial SS to be unrelated, as the starting urine SS has no apparent link to the amount of calcium or oxalate that urine can dissolve without leading to crystal formation. However, in rats, we have found a surprising correlation between ULM and SS, such that ULM appears to rise with initial SS, for CaOx, and, to a lesser extent, for brushite (Br), a typical calcium phosphate initial phase. In this study, we measured CaOx and Br ULM, and SS, in urine of 50 patients and 11 normal people, to determine if ULM and SS were correlated, as in rats, and to explore the relationship between SS and ULM. We found the same dependence of ULM on SS as in rats, for both CaOx and Br, and found no differences between patients and normal people with respect to this dependency. However, for Br, patients showed a lower ULM than normals, but the same initial SS, meaning that patients were closer to their crystal formation threshold than normals. Treatments for stones had no apparent effect on the SS-ULM dependency. We conclude that in humans, as in rats, ULM is related to initial SS, and that this relationship is the same in patients as in normals for CaOx, but shifted in a stone forming direction for Br among patients. The ULM-SS interaction is unaffected by contemporary conventional stone treatments, and is more marked for CaOx than Br. The mechanisms of the dependence are unknown. The smaller difference between ULM and initial SS for Br in patients than normal supports prior evidence suggesting a defect in stone patients that could lead to calcium phosphate crystallization, subsequent nucleation of CaOx, and stone disease.

Formation of renal stones requires production of solid phases such as calcium oxalate (CaOx), calcium phosphate, uric acid, struvite, and less common crystals [1]. Of these, CaOx and calcium phosphate are among the most common in human calculi [1]. The driving force for crystallization is supersaturation (SS), which can be expressed as the ratio of the concentration of the dissolved salt divided by the solubility of that salt in urine at body temperature [2]. As SS rises in urine, a point is reached at which solid phase begins to form, and that SS point is usually referred to as the upper limit of the metastable range (ULM) [3]. Below this limit SS permits growth of preformed crystals but not their *de novo* formation [3].

In principle, stone risk should follow the closeness of the SS of a urine to the ULM in that urine [4]. Such comparisons have been usefully performed using an experimental technique for determining the ULM, in which SS is raised progressively by addition of oxalate or calcium. As SS rises, the ULM is detected by observance of cloudiness from crystals [4–6]. When urine pH is fixed at 6.4, sodium oxalate addition results in the eventual crystallization of CaOx [5, 7], and the SS with respect to CaOx monohydrate at the crystallization point is called the ULM for CaOx. Adding calcium chloride at pH 6.4 causes eventual crystallization of brushite (Br, calcium monohydrogen phosphate) [5], and the ULM is usually expressed as SS with respect to Br at the crystallization point.

Using this valuable technology in the analysis of stone formation in a colony of inbred hypercalciuric rats [8], we established that high initial Br SS was combined with a Br ULM quite close to the initial SS. By contrast, the ULM for CaOx was much higher than the initial CaOx SS. A reasonable basis for the observed fact that such rats form calcium phosphate stones [9] could well be the much smaller distance between SS and ULM for Br versus CaOx. In the course of the work, we noted that ULM for CaOx and Br seemed related to their initial SS, a fact that was surprising and not easily explained. The present study was designed to test whether this linkage between ULM and SS could be found in humans. Accordingly, we measured ULM in normal people, and in both treated and untreated patients with nephrolithiasis.

METHODS

Patients and normals

Fifty-four 24-hour urine samples were collected from 50 patients (19 women); one man and one woman provided three pretreatment urines each. The rest each provided one urine sample. In all, 28 urines were pretreatment, and 26 were during treatment. Urines were collected unrefrigerated using thymol as a preservative [10]. Of the 50 stone formers, 39 had calcium stones, 9 had urate stones (8 admixed with calcium), 1 had cystine stones and 7 patients had unknown stone types. Metabolic abnormalities included hypercalciuria in 31 patients, hypocitraturia in 18 patients, hyperoxaluria in 8 patients, hyperuricosuria in 6 patients,

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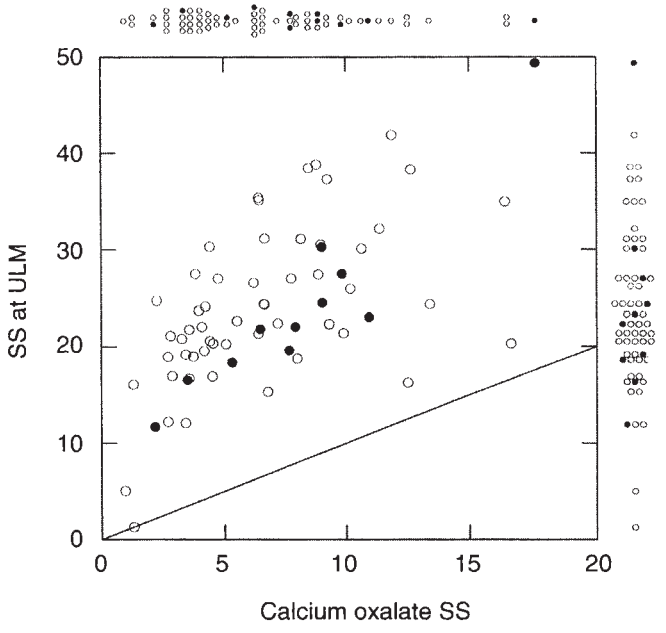

Fig. 1. Relationship between the upper limit of metastability (ULM) and supersaturation (SS) for calcium oxalate (CaOx). Among normals (●) and patients (○), ULM rose with SS. Values for the regression are in the **Results** section. All but one point is above the diagonal line of identity, indicating that ULM exceeds SS by a generous margin. Although the normal points seem at the low end of the patient distribution, normal ULM values were not lower even when covariance with SS was considered (Table 1). The general overlap of SS and ULM values for patients and normals is evident from the dot plots along the horizontal and vertical axes of the graph.

and cystinuria in one patient. The total for both stone type and metabolic abnormality exceeded the 50 patients since some patients had mixed stone types and multiple metabolic problems. Treatments [11] included hydration and reduced sodium and oxalate intake, and active medications including thiazide diuretic agents (4 patients), sodium or potassium citrate or bicarbonate salt supplements (9 patients), both agents together (12 patients), and allopurinol alone (1 patient). No patterns of difference emerged among the kinds of treatments, so treatment urines are pooled for analysis. Patients were unselected except for being the consecutive new and follow-up patients over a four month period. Eleven normal people (2 women) each provided one 24-hour collection.

Routine laboratory measurements including supersaturation

Calcium, chloride, creatinine, magnesium, sodium, potassium, phosphate, ammonium, and uric acid concentrations were measured in each urine by standard laboratory techniques (Beckman Synchron CX5; Beckman Instruments, Brea, CA, USA). pH was measured by glass electrode. Sulfate was measured by barium precipitation [12]. Oxalate was measured by enzyme assay using oxalate oxidase (Sigma Corp., St. Louis, MO, USA). Citrate was measured by enzyme assay using citrate lyase (Mannheim Bohringer, Mannheim Germany). From this array, we calculated the SS with respect to CaOx and Br using the relative saturation ratio from EQUIL2, an iterative computer program [2].

Table 1. Supersaturation (SS) and upper limit of metastability (ULM) values in patients and normals

	Normals (11)		Patients (54)	
	Unadjusted	Adjusted	Unadjusted	Adjusted
SS CaOx	8 ± 1		6.6 ± 0.7	
ULM CaOx	24 ± 3	22 ± 2	24 ± 2	24 ± 1
SS Br	1.4 ± 0.4		1.4 ± 0.2	
ULM Br	5.7 ± 0.6	5.7 ± 0.5	4 ± 0.2[a]	3.9 ± 0.2[b]

Differs from normal, [a]$P < 0.05$ [b]$P < 0.001$

Abbreviations are: unadjusted and adjusted refer to covariance with corresponding SS; SS, supersaturation; CaOx, calcium oxalate; Br, brushite a specific calcium phosphate phase (**Methods**); ULM, the value of SS at the point of visible crystal formation (**Methods**). All values are means ± SEM.

Upper limit of metastability

A modification of the method described by Nicar, Hill and Pak [5] was used to determine the ULM of CaOx and Br in human urine as previously described from this laboratory [8]. An aliquot of urine from a 24-hour urine collection was centrifuged for 30 minutes at 3000 RPM to remove debris. Urine pH was adjusted to 6.4 by addition of HCl or NaOH as required. Ten milliliters of each urine sample were placed into each of 13 tubes, and sodium azide was added to each tube, at a final concentration of 0.02%, to prevent bacterial growth. The tubes were placed in a water bath at 37°C and magnetically stirred. To initiate CaOx precipitation, increasing amounts of sodium oxalate were added to each set of tubes. One tube had no oxalate added to serve as blank. The pH of each tube was checked hourly during the incubation and acid or alkali added as required to maintain pH at 6.4. After three hours the samples were checked for visible precipitation; the tube with the lowest amount of oxalate added that initiated crystallization was considered the endpoint. The SS at the point of precipitation was calculated using EQUIL, assuming all chemical concentrations were unchanged except for oxalate, which was taken as the initial measured oxalate concentration plus the amount added to the tube. Brushite ULM was determined in the same fashion, except calcium chloride was added to the urine samples to precipitate Br [5].

Statistical analysis

Means and SD, *t*-tests, analysis of covariance, and interactive stepwise logistic regression were performed using standard statistical software (SYSTAT 6.0; SPSS Corp., Chicago, IL, USA).

RESULTS

Calcium oxalate ULM was highly correlated with CaOx SS (Fig. 1) among patients and normals ($r = 0.619$, $P < 0.001$). All but a few points rested far above the line of identity (diagonal line on the figure), showing the wide margin between SS and the ULM. This margin appears slightly lower in our normals than patients (compare closed to open symbols), but mean values for ULM in patients and normals did not differ significantly even when covariance with SS was accounted for (Table 1). If only the calcium stone formers are compared to normals, there was still no difference in CaOx ULM (24.1 vs. 23.6, $P = 0.904$). Among only patients, the relationship between ULM and SS remained strong

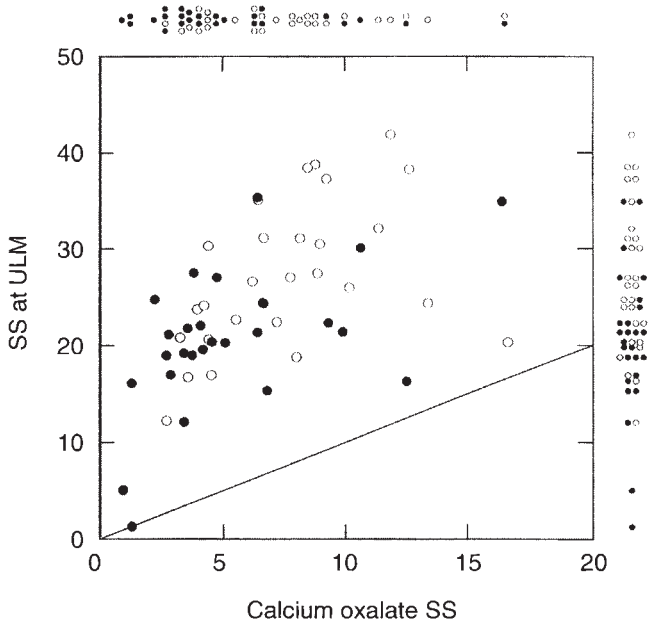

Fig. 2. Relationship between the upper limit of metastability (ULM) and supersaturation (SS) for calcium oxalate (CaOx) among treated (●) and untreated (○) patients. The ULM and SS were correlated, and occupied the same ranges (Table 2). Their intermixture is best visualized using the univariate plots along the axes of the graph.

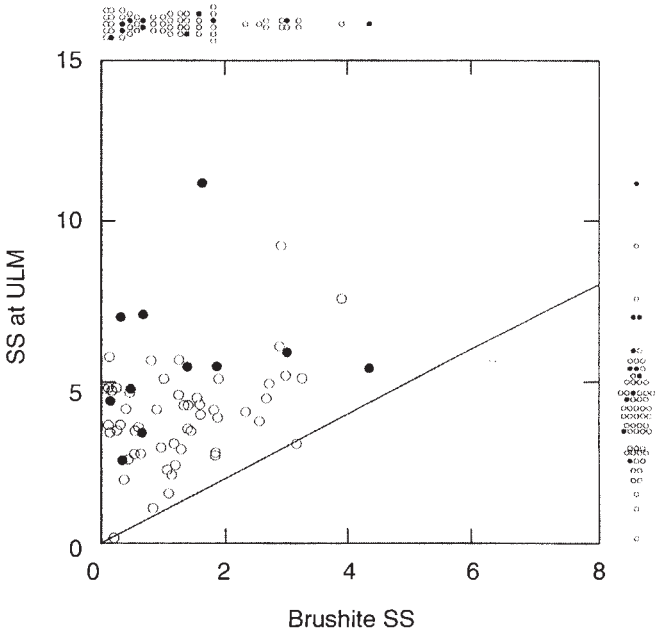

Fig. 3. Relationship between the upper limit of metastability (ULM) and supersaturation (SS) for brushite (Br). Among normals (●) and patients (○), ULM rose with SS. Values for the regression are in the **Results** section. Normal people have higher ULM values than patients, even when covariance with SS is considered (Table 1). This is best visualized using the univariate plot of ULM along the right axis of the graph. ULM exceeds SS among patients by a lesser amount than among normals.

Table 2. Supersaturation (SS) and upper limit of metastability (ULM) values in treated and untreated patients

	Untreated (28)		Treated (26)	
	Unadjusted	Adjusted	Unadjusted	Adjusted
SS CaOx	7.6 ± .7		5.4 ± 0.7[a]	
ULM CaOx	27 ± 2	26 ± 1	21 ± 1[b]	22 ± 1[a]
SS Br	1.33 ± 0.2		1.46 ± 0.2	
ULM Br	4.2 ± 0.3	4.24 ± 0.26[c]	3.7 ± 0.3	3.7 ± 0.27

Abbreviations are in Table 1.

Note that no comparisons were made between treated patients and normals.

Differs from untreated, [a]$P < 0.05$ [b]< 0.01; [c], differs from normal, $P < 0.02$

(Fig. 2) and uninfluenced (Table 2) by treatment versus pretreatment status (compare open vs. closed symbols). The regression of ULM on SS using only normal and pretreatment data from patients was also highly significant ($r = 0.625$, $P < 0.001$). Using only data from patients, the r and P values for pretreatment and treatment respectively were 0.44 and 0.018 versus 0.53 and 0.005. Urine collected during treatment had a lower CaOx SS than those collected pretreatment (Table 2); the comparison was between the treatment and pretreatment patient groups, not between treatment and pretreatment in individual patients. We did not compare treatment patient data to normal data, because the comparison seemed, to us, inappropriate.

For Br, ULM was correlated less dramatically with SS than was the case for CaOx (Fig. 3); however, the regression was statistically significant ($r = 0.340$, $P = 0.005$). Normals (closed symbols) had higher ULM values than patients, as shown by their position in Figure 3, and by both direct t-tests, and analysis of covariance (Table 1). Comparing only the calcium stone formers to the normals we found a significantly lower ULM for the stone formers (3.9 vs. 5.7, $P = 0.027$), but there was not a difference between the calcium stone formers and the stone forming group as a whole. Treatment status had no apparent effect on the relationship between ULM and SS (Fig. 4 and Table 2), even though the regression of ULM on SS using only normal and pretreatment data from patients was marginal ($r = 0.301$, $P = 0.058$). Regression coefficients (r) and P values for pre-treatment and treatment were 0.405, $P = 0.033$ and 0.538, $P = 0.005$, respectively. Using only pretreatment results, ULM no longer differed significantly from normal, but this was due mainly to the reduced sample size, as the difference of means was the same (Tables 1 and 2). Treatment ULM for Br (Table 2) was lower than pretreatment, but not significantly. Using SS as covariate, ULM also did not change significantly between treatment and pretreatment, but ULM Br was lower in patients than in normals (Table 2 vs. Table 1, adjusted ULM values). Brushite SS did not change significantly with treatment.

The ULM is correlated not only with SS, but also with other urine measurements (Table 3). Common to both CaOx and Br ULM were both the CaOx and Br SS values, and urine calcium and citrate concentrations. Urine pH, oxalate, and phosphorus concentrations were correlated with ULM for Br, but not for CaOx. These many correlations are difficult to make sense of because urine concentrations are themselves volume dependent, in part, and therefore correlated with each other and with SS itself. To clarify the independent covariates of ULM, we used

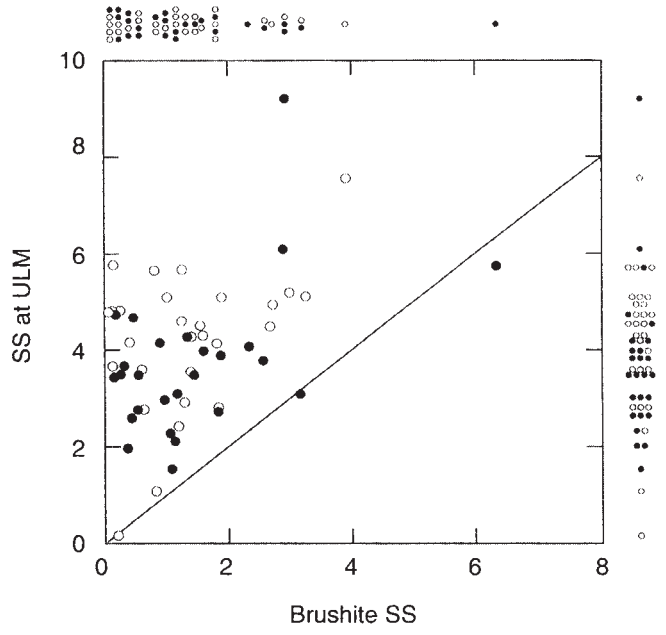

Fig. 4. Relationship between the upper limit of metastability (ULM) and supersaturation (SS) for brushite (Br) in treated (●) and untreated (○) patients. The modest and statistically insignificant effects of treatment on both variables can be visualized using the univariate plots along the axes of the graph. Without treatment, ULM is below normal (compare to Fig. 3, Table 2 to Table 1).

Table 3. Univariate correlations with the upper limit of metastability (ULM)

	ULM CaOx		ULM Br	
	r	*P*	*r*	*P*
SS CaOx	0.619	0.000	0.639	0.000
SS Br	0.259	0.037	0.340	0.005
Calcium	0.595	0.000	0.654	0.000
Citrate	0.398	0.000	0.434	0.000
pH	−0.22	NS	−0.57	0.000
Oxalate	0.197	NS	0.571	0.000
Phosphorus	0.224	NS	0.774	0.000
Sodium	0.16	NS	0.38	0.002
Potassium	0.011	NS	0.42	0.0004

Values of *P* are for the individual measurements, *r*, the regression coefficient.

Abbreviations are in Table 1.

Table 4. Multivariate correlation of the upper limit of metastability (ULM) on urine measurements

ULM CaOx	*r*	*P*	ULM Br	*r*	*P*
SS CaOx	0.625	0.000	Phosphorous	0.723	0.000
Oxalate	0.735	0.0014	Citrate	0.782	0.0061
Citrate	0.796	0.0044	pH	0.811	0.032

Regression was performed using pretreatment values from patients and the 11 normals as a pool. Values of *r* are cumulative as each new term enters; *P* values are for the partial correlation of the measurement with ULM in the final ensemble of three measurements in the total regression. All measurements were in mg/dl, except pH and SS which are unitless. For ULM Br, a slightly better overall regression ($r = 0.825$) could be obtained with SS Br in place of phosphorus. Coefficients for the final CaOx regression were 2.08 ± 0.26, 0.12 ± 0.04, and -5.48 ± 1.1 for SS CaOx, urine citrate, and urine oxalate (latter two in mg/dl), respectively. For ULM Br, coefficients were 0.032 ± 0.006, 0.02 ± 0.007 and -0.737 ± 0.332 for urine phosphorus and citrate concentrations (in mg/dl), and pH, respectively.

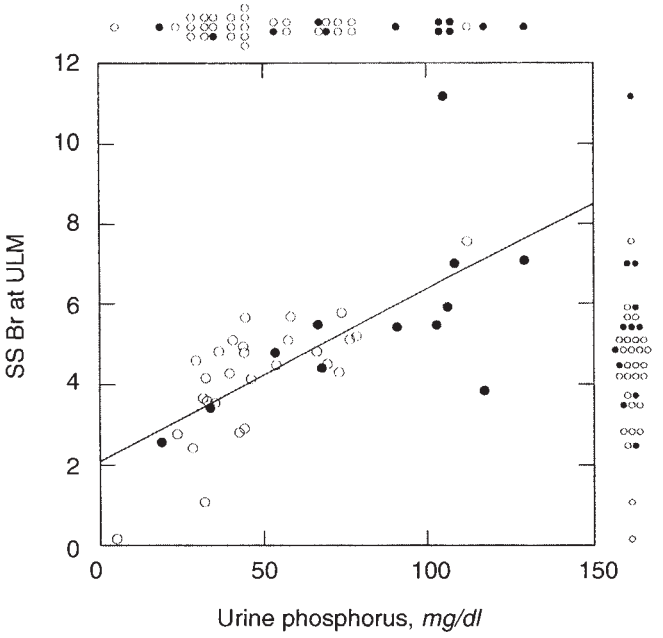

Fig. 5. Relationship between brushite (Br) upper limit of metastability (ULM) and urine phosphorus concentration. Among pre-treatment patients (○) and normals (●) the ULM (y axis) varied smoothly with urine phosphorus concentration (x axis). The line of linear regression is shown ($r = 0.774$, $P < 0.001$, Table 3; multivariate values in Table 4).

interactive stepwise logistic regression, with forward stepping. For CaOx ULM (Table 4), CaOx SS and urine oxalate and citrate concentrations were the independent covariates, whether we considered all patient samples and normals, or normals with only pretreatment patient samples. Urine volume itself had no independent effects in the multivariate analysis.

For ULM Br, urine phosphorus and citrate concentrations, and urine pH were the independent correlates when all samples were considered. When only pretreatment patient samples and normals were considered, equivalent fits were obtained with either urine phosphorus concentration (as in Table 4) or with SS Br in place of urine phosphorus concentration, along with urine citrate and pH. Our study offers no direct information concerning possible mechanisms of linkage between ULM and SS or other urine measurements; however, the phosphorus correlation is extraordinary (Fig. 5), suggesting it may well be of critical importance.

Since CaOx SS is strongly dependent upon urine oxalate concentration, the independent effect of urine oxalate concentration itself upon ULM CaOx (Table 4), along with SS, suggests that urine oxalate concentration is somehow itself able to influence ULM apart from SS. The same is true for urine citrate concentration. The nature of the oxalate effect is a reduction of ULM for a given CaOx SS, that creates a significant fraction of the scatter in Figure 1 (Fig. 6). Here, the plot of Figure 1 is reproduced, except that the size of each symbol is scaled to urine oxalate concentration and the line of identity is replaced by a power

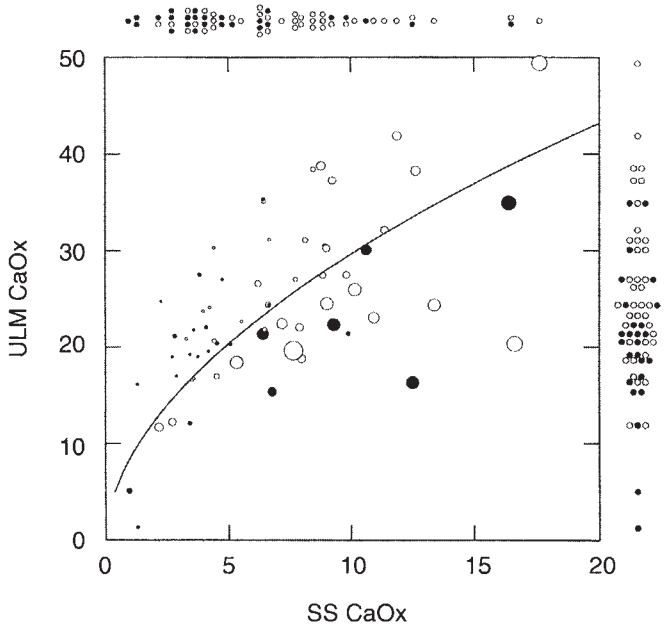

Fig. 6. Interaction between the upper limit of metastability (ULM), calcium oxalate (CaOx) supersaturation (SS), and urine oxalate concentration. The ULM (y axis) varies with SS (X axis) as in Figure 1; the powered regression line ($y\text{-}ax^b$) is shown here instead of the line of identity of Figure 1, otherwise the 26 treatment urines (●) and results from 28 pretreatment samples and 11 normal samples (○) are identical to Figure 1. Symbol size is proportioned to urine oxalate concentration. Note the striking preponderance of large symbols below the regression line, which graphically depicts the negative correlation of ULM on urine oxalate concentration (Table 4).

function regression line. The lower ULM points, which lie below the regression line, contain the majority of large symbols, which depict higher urine oxalate concentrations. These are evenly distributed between treatment (light fill) and pretreatment and normal samples (dark fill). If a similar plot is made using citrate concentration, higher values lie above the regression, but to a lesser extent, however (not shown).

DISCUSSION

The apparent linking of ULM to SS for both CaOx and Br is surprising, and rather difficult to explain. That it occurs in rats [8] and humans, and in normals and patients, treated and untreated alike suggests that it is an important phenomenon. It is not likely to be due to an *in vitro* artifact as we used a well established method to find values for ULM not very different from those described by other researchers [5]. In simple salt systems, the point of crystallization has no relationship to the arbitrary starting SS of the fluid; whatever the initial SS, provided it is below the ULM, one simply raises the SS to the ULM.

The linkage phenomenon is more obvious for CaOx than Br, though statistically both show strong regressions of ULM on SS. The larger gap between the SS and the ULM, for CaOx versus Br, observed by others in humans [4] and by us in rats [8] makes CaOx seem less likely to form, even though CaOx is certainly the most prevalent stone salt in humans. It may be that CaOx is not the most common or important initial solid phase, but a very stable phase that occurs as part of a cascade of nucleation events that begins with another solid phase, perhaps a calcium phosphate. We might presume that CaOx is prevalent because it is stable at all pH values urine can attain, whereas the calcium phosphate phases are not stable at pH values much below 6 [13].

Normal people do not have higher CaOx ULM values than patients, and treated patients are no higher than untreated. However, Br ULM values are higher in normals than patients, as is evident statistically and by inspection of Figure 3. This discrepancy arises from unknown sources, but suggests some possible defect in a normal protective system among patients that would lead to Br crystallization. Given the large body of evidence that calcium phosphate crystal can be an efficient heterogeneous nuclei for CaOx [14–17], and that Br is an early nucleating phase in a sequence that eventuates in apatite [7, 18, 19], it is possible that the lower ULM for Br might be important in producing CaOx stones. In this case, the lack of a reduced CaOx ULM in patients may well reflect Br or another calcium phosphate phase acting as a nucleator for CaOx. Therefore, CaOx would not have to achieve its own ULM in urine in order to become a stone solid phase.

Using the same experimental technique, but a different approach to calculation, Pak and Holt [6] found reduced Br ULM in patients versus normals, accompanied by higher Br SS in patients. The correspondence of our Br ULM results with theirs add confidence that both are correct. Pak and Galosy [4] also report reduced ULM for Br using many groups of patients with nephrolithiasis. By contrast, both prior studies find lower CaOx ULM among patients than normals, whereas we did not. Moreover, the strong positive correlation we find between SS and ULM is contrasted to the low CaOx SS and high ULM they found in normals versus patients. That we agree in one and not the other solid phase is unexplained at this time. Differences in patient groups cannot be excluded.

The possible mechanisms linking ULM and SS are unclear, but several remarkable correlations suggest possible avenues for subsequent exploration. ULM for CaOx is clearly inversely related to urine oxalate concentration at any level of CaOx SS, even though urine oxalate is itself a major component of CaOx SS and CaOx SS is strongly and directly proportional to urine oxalate concentration. Perhaps the usual high calcium to oxalate ratio of urine (typical values are 2 to 8 mM for calcium and 0.2 to 0.5 mM for oxalate) [13] reduces crystallization and a higher urine oxalate, by bringing the ratio closer to equimolarity, fosters nucleation, lowering the ULM. Unbalanced stoichiometry could possibly explain the strong and independent proportional relationship between CaOx ULM and SS CaOx. If we plot the ULM against the ratio of calcium to oxalate molarity (Fig. 7) high and low SS points, indicated by size variation, scatter on both sides of the linear regression line. However, SS, ULM, the calcium:oxalate ratio, and oxalate molarity all are strongly interrelated, so statistical inferences are very tenuous. For example, if SS CaOx is taken in stepwise regression as a correlate of ULM CaOx, the calcium to oxalate molar ratio remains highly significant ($F = 6$, $P < 0.01$), but oxalate molarity is more so ($F = 12$, $P < 0.001$); if oxalate molarity is also taken as a second covariate with ULM, the ratio loses all significance ($F = 0.2$, $P = 0.655$). In other words, new experiments must elucidate the matters that our analysis can only suggest may be important.

Brushite ULM is related most strongly to urine phosphorus concentration (Fig. 5). Perhaps urine pyrophosphate, which varies

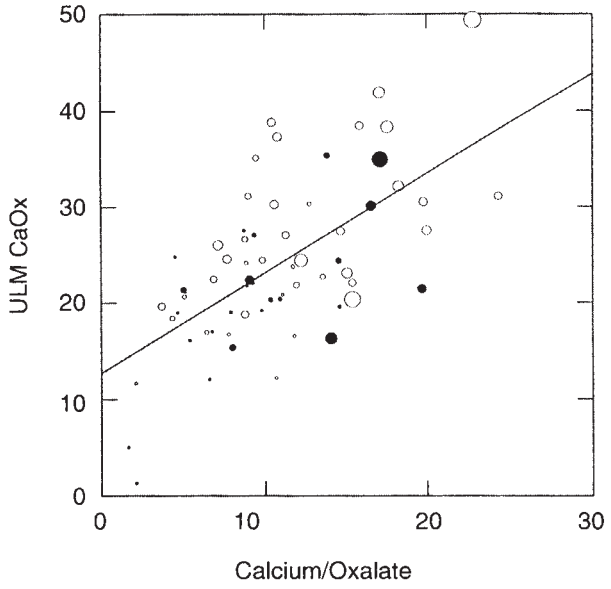

Fig. 7. Interaction between the upper limit of metastability (ULM) calcium oxalate (CaOx) and the molar calcium to oxalate concentration ratio. The ULM varied strongly with the ratio (x axis), as it did with CaOx SS; however, the ULM values for samples with high CaOx SS (large symbols) scattered symmetrically about the linear best fit regression, compatible with the assumption that the correlation between ULM and CaOx SS was due in significant measure to variation of the molar ratio.

proportionately to urine total phosphorus concentration, is important in elevating the Br ULM [20, 21]. This in turn may explain the relationship between SS Br and ULM Br, since SS Br is directly and strongly related to urine phosphorus concentration. As well, other inhibitors, large or small molecules, could play an as yet unspecified role.

The clinical relevance of this study is as yet unexplored, but has considerable potential. Perhaps the relationship between ULM and SS is a determining feature of stones, as others have suggested [4–7, 22–25]. Of note, prior workers [4–7] did not find any dependence of ULM on SS for either CaOx or Br. However, when urine volume and calcium intake are not controlled, as they were in prior studies [4–7], and the ranges of SS and perhaps the molar calcium to oxalate ratio are allowed to become large, as in this study, ULM varies with SS, most markedly for CaOx. Defects in patients could reside not only in the difference between ULM and SS, but in the response of ULM to SS, especially for CaOx. For Br, the correspondence of prior clinical studies with this one, and with studies in hypercalciuric rats, makes a low ULM for Br seem rather characteristic of both calcium phosphate stone forming rats and humans who make predominantly CaOx stones. The prevalence of this apparent defect seems high, and patients with it may turn out to have hitherto unsuspected defects in other aspects of urine chemistry or inhibitor molecules.

Finally, our study may prompt some to ask how crystals form in the first place, given ULM values above SS in almost all samples and—perhaps even more fundamentally—if crystals are present in urine, how SS is maintained. About the former, we can only speculate. Perhaps spikes of SS, averaged out by 24 hour collections, are not accompanied by corresponding ULM, and permit nucleation. It may well be that Br and CaOx are not the only relevant solid phases, and some other phase, probably a poorly formed apatite [26] has a ULM near to average SS, which is easily exceeded. Perhaps seed crystals form in a part of the nephron whose SS is not reflected in the final urine, and these nucleate CaOx, in a cascade. We have in fact proposed such SS in the thin limb of the loop of Henle [26]. About the latter, we are reasonably confident that the universal SS of urine with CaOx reflects kinetic retardation by urine molecules [15]. Crystals certainly are present in urine of stone formers and many normals [3], so without such retardants ions would be depleted over hours to a few days [3]. With them, the process is delayed by 10- to 100-fold, and urines collected and processed within a few days will show stable SS values.

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APPENDIX

Abbreviations used in this article are: SS, supersaturation; ULM, upper limit of metastability; CaOx, calcium oxalate; Br, brushite.

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